Luteal Blood Flow and progesterone concentration during first and second *postpartum* estrous cycle in lactating dairy cows.

Monitoring ovaries by Power Doppler in the bovine

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Abstract

The aim of the present study was to determine the differences in corpus luteum (CL) functionality between the first *postpartum* estrous cycle and the following cycle in lactating dairy cows. Luteal blood flow (LBF), luteal size and blood progesterone (P4) concentration were monitored during the first and second *postpartum* estrous cycle. During the first and second *postpartum* estrous cycle, the mean LBF value increased ($P<0.05$) from early to late diestrus, while it decreased rapidly in proestrus, resulting statistically lower ($P<0.05$) than those registered in all previous phases. Statistically significant differences were not observed between overall LBF during first and second *postpartum* estrous cycle ($P>0.05$). During the first *postpartum* estrous cycle, P4 blood concentrations showed a significant reduction ($P<0.05$) from diestrus to proestrus. A different trend of P4 concentrations was observed during the second *postpartum* estrous cycle, where mean P4 value registered in proestrus resulted statistically lower than those registered in the previous cycle phases ($P< 0.05$). The mean P4 concentration registered over the first *postpartum* estrous cycle resulted statistically lower ($P<0.05$) than that registered during the second one. A significant correlation between P4 concentrations and LBF was registered only during the second *postpartum* estrous cycle. Results indicate that during the first *postpartum* estrous cycle P4 concentration was independent of luteal blood flow and luteal size.

Key words: dairy cow; *postpartum*; power Doppler; corpus luteum

Introduction

Reproductive performance is one of the important factors determining the profitability of dairy herds, but increased milk yield in dairy cattle determine a decline in their fertility (Wiltbank et al., 2002). The study of *postpartum* and the monitoring of the recovery of ovarian activity are important elements for dairy farmer. An increase in the proportion of cows conceiving soon after the elective waiting period will decrease the proportion of cows with extended lactations, that are less profitable (Ribeiro et al., 2012). The increase in genetic merit for milk production over the past decades has been associated with an overall decrease in reproductive performance of dairy cows (Lucy, 2001). Indeed, the increased dry matter intake, liver blood flow and greater metabolic clearance rate in the *postpartum* period are associated with reduced peripheral concentrations of steroid hormones such as estradiol and progesterone in high-producing cows (P4; Sangsritavong et al., 2002). The reduced blood hormones concentrations affect the hypothalamus-pituitary-ovarian axis (Wiltbank et al., 2006) and uterine physiology of the cow (Geisert et al., 1992) and could explain the poor reproductive performance reported in modern dairy herds. Usually, the first *postpartum* period is characterized by a physiological anestrous status that lasts about 15-20 days (Wiltbank et al., 2002). As reported by different Authors, the first *postpartum* estrous cycle presents lower duration and fertility than the subsequent cycles, because of an early corpus luteum (CL) regression, determined by an untimely prostaglandin secretion by the uterine glands (Kozicki et al., 1998; Inskeep and Dailey, 2004). The inefficiency of CL endocrine activity during the first *postpartum* estrous cycle is evidenced by low milk (Kozicki et al., 1998) and blood (Kayacik et al., 2005) progesterone concentrations.

Corpus Luteum is one of the most highly vascularized organs; it receives the greatest blood flow per tissue volume in the body (Wiltbank et al., 1989). In the first week after ovulation, blood vessels from the theca interna invade into the follicular cavity and form a network, which supplies luteal cells. This neovascularization is necessary for provision of low-density lipoprotein, used by luteal cells for progesterone (P4) biosynthesis, and for the delivery of luteal steroids to circulation (Carr et

al., 1982). Furthermore, luteal endothelial cells secrete vasoactive substance, such as nitric oxide, endothelin-1, angiotensin-II or prostaglandins, directly involved in P4 secretion. Therefore, both endothelial cells and blood vessels of CL play a crucial role in its functionality (Miyamoto et al., 2009). Since luteal vascularization is very important for the CL function, the study of luteal blood flow (LBF) give a valuable information about it (Miyamoto et al., 2009). Until the advent of Doppler technology, vascularization of the bovine ovaries was investigated experimentally using invasive procedures (Bollwein et al., 2013). In the past 15 years, Doppler technology has replaced invasive techniques for monitoring of bovine reproductive system (Ford et al., 1979). Since blood vessels of the CL have a very low blood flow velocity, Color Power Doppler ultrasonography, a noninvasive diagnostic method detecting the number of red cells moving through vessel per time unit and showing them as colored pixels, is the most advantageous method for evaluating Luteal Blood Flow (LBF) (Bude et al., 1994). Despite several Authors examined LBF during different stage of bovine estrous cycle (Vasconcelos et al., 2001; Shirasuna et al., 2004; Miyamoto et al., 2005; Herzog et al., 2010; Lüttgenau et al., 2011) or for early pregnancy diagnosis (Utt et al., 2009; Siqueira et al., 2013; Kanazawa et al., 2016), no data have been reported on LBF in *postpartum* dairy cows. Therefore, the aim of the present study was to compare luteal competency by serum progesterone concentrations and LBF, measured by Power Doppler technique, during the first and second *postpartum* estrous cycle in Holstein Friesian cows.

Materials and methods

Animals

This study was conducted on lactating dairy Holstein cows housed at the farm of Department of Veterinary Medical Sciences, University of Bologna. All experimental procedures were approved by the University of Bologna Ethical Review Committee and the Ministry of Health.

An anamnestic investigation and a complete physical examination were performed before starting. Only cows between 2 and 5 years old, 15 days after delivery were examined. The average number of calving per cow was 2 (range 1-3) (Table 1). To remove any influence of disease on the ovarian conditions, cows with BCS<2.5 (Body Condition Score: scale 1 to 5, with 0.25-point increments; Ferguson et al., 1994), history of caesarean section or dystocia, retained foetal membranes, vaginal laceration or severe systemic diseases were excluded from this study, as well as cows treated with systemic antibiotic therapy or intrauterine therapy before enrollment. In total, 14 cows were examined. Four animals developed endometritis during the experiment so were excluded, since it was demonstrated that reproductive and systemic diseases could influence LBF and P4 blood concentrations (Strüve et al., 2013). Furthermore, 4 cows developed other pathologies and were excluded from the study (follicular cyst; luteal cyst; persistent corpus luteum; ketosis), leaving only 6 cows eligible for inclusion in the experiment.

Animals were housed in curtain-sided free-stall barns and fed a total mixed ration based on alfalfa/grass hay (50% ration dry matter), corn, barley and protein supplements. The cows were non-seasonal, year-round calves, milked twice daily with herd average 305-days milk yield around 9000-10.000 Kg per cow.

Luteal blood flow and Luteal area assessment

In order to identify the precise moment of ovarian activity resumption, the investigation of internal genital structures was carried out by trans-rectal palpation and ultrasound examination (5MHz linear probe, Tringa Lineare Vet, ©2012 Esaote S.p.A., Milan), twice a week, starting from Day 15 after calving. All sonographic investigations were conducted by the same operator; during manipulations, cows were at the feeding rack and no animal was sedated.

For all enrolled cows, during the first and second *postpartum* estrous cycle, ultrasound examinations were ideally (considering a standard 21 days cycle) carried out to follow CL function

on Day 6-7 (Cycle phase 1 - early diestrus), 9-10 (Cycle phase 2 - diestrus), 14-15 (Cycle phase 3 - late diestrus) and 17-end of the cycle (Cycle phase 4 - regressing CL, proestrus) (Day 0=ovulation). Power-flow Doppler (5 MHz linear probe, MicroMaxx ® SonoSite Inc. Bothell, WA) was used for luteal blood flow mapping. Care was taken to locate the entire CL transverse section within the Doppler sample box, in order to avoid flash alterations and to evaluate maximal blood flow within the CL. At least three images without flash artifacts and with a maximum number of colored areas were recorded. The analysis of the stored Doppler images was carried out using an image processing software (ImageJ-2; National Institutes of Health, USA). The entire luteal structure and its blood flow area were separated from the rest of ovarian tissue, and colored area within this region was calculated. The area of detectable CL blood flow is expressed as a percentage of the CL area and it was calculated by the application of the following ratio:

tot pixel: 100 = color pixel: X

where X represents the percentage of vascularized CL (Ginther et al., 2004). The mean of the three single images was calculated and used for statistical analysis.

For luteal area assessment, three cross-sectional images with maximal areas were recorded and analyzed using a computer-assisted images analysis software (ImageJ-2; National Institutes of Health, USA).

Hormone analysis

Immediately after ultrasonographic examinations of each animal, blood samples were collected from the coccygeal veins into evacuated tubes (4.9 mL test-tube Monovette ® Serum gel, Sarstedt, Germany). Serum was separated by samples centrifugation at 1500 x *g* for 10 minutes and then stored in a 1.5 mL test-tube (Sarstedt, Germany) at -80 C° until the hormone assays were performed. Serum progesterone levels were assessed by an enzyme immunoassay (IMMULITE® Immunoassay System, Siemens Health Care and Diagnostic Inc., Gweynedd, UK). This is a

sequential competitive immunoassay system characterized by two incubation cycles (1 x 30 min). The lower detection limit was 0.2 ng/mL.

Statistical analysis

After knowing the length of every single estrous cycle, data collected were proportionally (considering the supposed phases during an ideal 21 days cycle) positioned in the right phase of that cycle. Data were analyzed for normality using a Shapiro-Wilk test. Milk production, cycle length, P4 and LBF levels in the single phases were compared between cycles using a paired Student T-test or a Wilcoxon test. Statistical differences in P4 and LBF levels over time were assessed by repeated measure GLM or a Friedman test, using a Tukey HSD test for post hoc comparison or a Wilcoxon-Mann-Whitney test. A Pearson test was used for analysis of P4, LBF and luteal area correlations. All tests were performed using IBM SPSS Statistics 25 (IBM Corporation, Milan, Italy). For all analyses, $P<0.05$ was considered significant.

Results

Enrolled cows had the first ovulation between 18 and 50 days after calving (mean value: 29.7 ± 11.7 days after calving). No statistically significance differences ($P>0.05$) were found between first and second *postpartum* estrous cycle in milk production (37.7 ± 4.3 vs 38.8 ± 4.3, respectively) and cycle length (18.3 ± 5.2, range 10-26 days vs 23.8 ± 5.8, range 15-30 days, respectively).

LBF trend in the first and second estrous cycle is showed in Figure 1. Mean LBF values registered in single cycle phases were similar during the first and the second *postpartum* estrous cycles ($P>0.05$; Table 2).

During the first *postpartum* estrous cycle, the mean LBF value increased ($P<0.05$) from phase 1 to phase 3 (12.1 ± 5.1 vs 23.3 ± 13.9 %), while it decreased rapidly in cycle phase 4, resulting statistically lower ($P<0.05$) than those registered in all previous phases (Table 2 and Figure 1). The same trend of LBF was observed also during the second *postpartum* estrous cycle. Indeed LBF increased ($P<0.05$) from cycle phase 1 to cycle phase 3 (12.2 ± 6.5 % vs 22.7 ± 6.3 %), while the

mean value registered in cycle phase 4 was the lowest ($P<0.05$; Table 2; Figure 1). Statistically significant differences were not observed between overall LBF during first and second *postpartum* estrous cycle ($P>0.05$).

During the first *postpartum* estrous cycle, P4 blood concentrations showed a significant reduction ($P<0.05$) from cycle phase 2 to 4 (3.7 ± 1.3 ng/mL vs 0.85 ± 0.71 ng/mL) (Table 2 and Figure 2). A different trend of P4 concentrations was observed during the second *postpartum* estrous cycle, where mean P4 value registered in cycle phase 4 (0.4 ± 0.15 ng/mL) resulted statistically lower than those registered in the previous cycle phases ($P< 0.05$) (Table 2; Figure 2). Blood progesterone concentrations measured during the second estrous cycle were not statistically different ($P>0.05$) than those registered during the same phases of the first *postpartum* estrous cycle. However, the mean blood P4 concentration registered over the first *postpartum* estrous cycle resulted statistically lower ($P<0.05$) than that registered during the second *postpartum* estrous cycle (Table 2 and Figure 2).

Luteal mean area registered during the first and the second *postpartum* estrous cycles, expressed in pixels, are reported in Table 3; no statistically significant differences were registered between the two considered estrous cycles ($P>0.05$). Pearson test showed only a significant ($P<0.05$) correlation between total blood progesterone concentrations and total LBF registered during the second *postpartum* estrous cycle ($R = 0.692$; Table 3). In both estrous cycles studied in the present work, no correlations were found between LBF and luteal area neither between P4 blood concentrations and luteal area ($P>0.05$; Table 3).

Discussion

While transitioning from late gestation to early *postpartum*, high-producing dairy cows experience profound metabolic changes involving regulation of energy status, liver function, mammary gland demand for glucose as required for lactation (Drackley, 1999). These changes are often linked to abnormal ovarian processes associated with poor reproductive performance (Wiltbank et al., 2006).

It can also be confirmed in the present study by the number of excluded animals from enrolled cows (8/14). The presence of atypical estrous cycles early *postpartum* is associated with reduced fertility (Lamming and Darwash, 1998). Therefore, the evaluation of ovarian function may enhance the understanding of the declining trend in fertility in the high-producing dairy cow (Norman et al., 2009). As previously reported (Kawashima et al., 2006), cows enrolled in the present study ovulated within three weeks *postpartum* and milk yield is not different between first and second *postpartum* estrous cycle (Sakaguchi et al., 2004; Lüttgenau et al., 2011). The duration of the first and second *postpartum* estrous cycle registered in this study are similar to those reported for higher number of animals (Townson et al., 2002). It is likely that the number of finally enrolled cows was not enough to obtain a statistically significant difference between the length of the two examined cycles. Usually the second ovulation occurs after a short luteal phase: before the first *postpartum* ovulation, low concentrations of preovulatory estradiol may result in early generation of a luteolytic mechanism (Mann and Lamming, 2000). This may indicate that luteal activity was compromised during the first ovarian cycle compared with that of the second cycle. Our results confirm this hypothesis, since mean P4 concentration during the first *postpartum* cycle is lower than in the second, as already reported in previous studies (Kawashima et al., 2006; Rutter and Randel, 1984).

The normal development of CL and its capability to produce progesterone, growth and angiogenic factors and vasoactive substances depends on its vascularization. After ovulation, CL develops from the wall of ruptured follicle and it is characterized by highly active vascularization and repeated mitosis of steroidogenic cells (Acosta and Miyamoto, 2004). The intensity of this process reaches a peak 2-3 days after ovulation (Reynolds et al., 2000). In the past 15 years, Doppler ultrasonography has become one of the most important techniques for determining the LBF area to assess luteal function (Matsui and Miyamoto, 2009). In the present study, in both estrous cycles considered, the mean LBF value increased from cycle phase 1 (early diestrus) to cycle phase 3 (late diestrus). Lei et al. (1991) demonstrated, by a histological examination, that in bovine CL the vascular space increased by 25% between days 6 and 12 after ovulation, whereas the non-luteal cells decreased

throughout that period by 15%. Thus the increased LBF seems to be determined by vasodilation of existing arterioles and not by further vascularization. Shirasuna et al. (2004) suggested that $PGF_{2\alpha}$ release by the uterus stimulates nitric oxide production in the arterioles of the peripheral vasculature of the mature CL, determining vasodilatation. This could explain why in both estrous cycles the LBF keeps growing until late diestrus (cycle phase 3) while it decreases in proestrus (cycle phase 4). However, despite the decrease in LBF occurs in parallel with the decrease in P4, in the present study a positive correlation between LBF and P4 concentration was registered only in the second *postpartum* estrous cycle. P4 levels in the first cycle decline significantly after phase 2 while in the second cycle after phase 3, highlighting a shorter luteal activity in the first cycle and demonstrating that P4 concentrations, particularly in the mid-luteal phase, is independent of luteal blood flow (Lüttgenau et al., 2011). Furthermore, during the first and second estrous cycle no correlations were found between P4 levels and luteal size. Therefore, lower P4 concentrations registered during the first *postpartum* estrous cycle are not related to the amount of luteal tissue neither to its vascularization.

In *postpartum* dairy cows the increased feed intake and milk yield increases the hepatic blood flow and metabolic clearance rate of estradiol and P4, reducing their circulating concentrations (Wiltbank et al., 2006). Particularly, the reduced P4 might increase LH pulse frequency (Stock and Fortune, 1993), overexposing the pre-ovulatory follicle to lower intensity LH pulses (Wiltbank et al., 2006). This overexposure to LH pulses may mature the oocytes earlier, compromising oocyte quality and delaying ovulatory events in the early postpartum period (Wiltbank et al., 2006). Recently, Bruinjè et al (2017), have demonstrated that later commencement of luteal activity in *postpartum* dairy cows is associated with lower conception rates, increased days open, higher embryonic mortality, and required more veterinary interventions (suggesting more health or reproductive disorders) than those having normal activity. Apparently, there is possibly a link between health or metabolic disorders (Santos and Rutigliano, 2009; Vercouteren et al., 2015), compromised ovarian activity (Opsomer et al., 2000), and subsequent fertility in dairy cows.

In this way, the lower fertility registered by farmer in cows inseminated at the first *postpartum* estrous cycle is determined by lower blood P4 concentrations not related to CL dimension or vascularization but more likely to a reduced luteal tissue functionality. As already supposed by other Authors, the present study confirm that the P4 blood levels could be used as benchmarks in herds monitoring fertility.

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Conflict of interest

There was no conflict of interest that could be perceived as prejudicing impartiality of the research reported.

Authors contributions

Eleonora data designed the study, collected the data and drafted the manuscript. Martina Lucci collected the data. Gaetano Mari paper revision. Barbara Merlo designed the study, analyzed data and reviewed the manuscript.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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402 Table 1. Data registered for enrolled cows.

Cow	Age (years)	N° Calving	BCS
1	5	3	2.75
2	4.5	3	3
3	3.5	2	2.25
4	3	2	2.75
5	2.5	1	2.5
6	3.5	2	2.5
Mean ± SD	3.7 ±0.9	2.2 ±0.8	2.6 ±0.3

403

404 Table 2. Mean values of blood progesterone concentration and LBF on phase 1 (early diestrus), 2

405 (diestrus), 3 (late diestrus), 4 (proestrus) of the first and second *postpartum* estrous cycle.

Cycle phase	First oestrus cycle		Second oestrus cycle	
	P4 (ng/mL) (Mean±SD)	% LBF (Mean±SD)	P4 (ng/mL) (Mean±SD)	% LBF (Mean±SD)
1	2.5±0.8	12.1±5.1	3.5±1.3[a]	12.2±6.5
2	3.7±1.3[a]	18.7±9.9	4.8±1.4[a]	20.0±14.5
3	2.3±1.4	23.3±12.6	3.9±0.6[a]	22.7±6.3
4	0.8±0.5[b]	8.8±5.4	0.6±0.1[b]	8.6±2.4
Mean±SD	2.4±1.5[c]	15.9±11.0	3.3±1.9[d]	16.2±10.4

406 In the same column a vs b are significantly different ($P<0.05$); in the same line c vs d are

407 significantly different ($P<0.05$).

408

409 Table 3. Mean values of Luteal area (pixels), LBF and blood progesterone concentration of the first

410 and second *postpartum* estrous cycle. The values are expressed as Mean±SD.

Values	First oestrus cycle	Second oestrus cycle
P4 (ng/mL)	2.4±1.5	3.3±1.9*
% LBF	15.9±11.0	16.2±10.4*
Luteal area (pixels)	10416.0±5912.0	14732.5±7113.6

411 *significant positive correlation ($P<0.05$): $R=0.692$
412

413

Figure Legend:

Figure 1. Mean values of LBF during the first and second *postpartum* estrous cycle.

Figure 2. Mean values of blood progesterone concentration registered during the first and second *postpartum* estrous cycle.